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Hypusine Is Required for a Sequence-specific Interaction of Eukaryotic Initiation Factor 5A with Postsystematic Evolution of Ligands by Exponential Enrichment RNA*

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Aiguo Xu‡ and Kuang Yu Chen‡§¶

From the ‡Department of Chemistry, Rutgers-The State University of New Jersey and §The Cancer Institute of New Jersey, Piscataway, New Jersey 08854-8087

Hypusine is formed through a spermidine-dependent posttranslational modification of eukaryotic initiation factor 5A (eIF-5A) at a specific lysine residue. The reaction is catalyzed by deoxyhypusine synthase and deoxyhypusine hydroxylase. eIF-5A is the only protein in eukaryotes and archaebacteria known to contain hypusine. Although both eIF-5A and deoxyhypusine synthase are essential genes for cell survival and proliferation, the precise biological function of eIF-5A is unclear. We have previously proposed that eIF-5A may function as a bimodular protein, capable of interacting with protein and nucleic acid (Liu, Y. P., Nemeroff, M., Yan, Y. P., and Chen, K. Y. (1997) *Biol. Signals* 6, 166–174). Here we used the method of systematic evolution of ligands by exponential enrichment (SELEX) to identify the sequence specificity of the potential eIF-5A RNA targets. The post-SELEX RNA obtained after 16 rounds of selection exhibited a significant increase in binding affinity for eIF-5A with an apparent dissociation constant of 1×10^{-7} M. The hypusine residue was found to be critical for this sequence-specific binding. The post-SELEX RNAs shared a high sequence homology characterized by two conserved motifs, UAACCA and AAUGUCACAC. The consensus sequence was determined as AAAUGUCACAC by sequence alignment and binding studies. BLAST analysis indicated that this sequence was present in >400 human expressed sequence tag sequences. The C terminus of eIF-5A contains a cold shock domain-like structure, similar to that present in cold shock protein A (CspA). However, unlike CspA, the binding of eIF-5A to either the post-SELEX RNA or the 5′-untranslated region of CspA mRNA did not affect the sensitivity of these RNAs to ribonucleases. These data suggest that the physiological significance of eIF-5A-RNA interaction depends on hypusine and the core motif of the target RNA.

Eukaryotic initiation factor 5A (eIF-5A),[1] ubiquitously present in eukaryotes and archaebacteria, but not in eubacteria, is the only protein known to contain a hypusine residue (for review, see Refs. 1–3). Hypusine is formed in two steps: (i) deoxyhypusine synthase catalyzes the transfer of a 4-aminobutyl moiety from spermidine to a specific lysine residue to form a deoxyhypusine residue, N^{ϵ}-(4-aminobutyl)lysine; and (ii) deoxyhypusine hydroxylase catalyzes the hydroxylation of the deoxyhypusine residue to form hypusine (N^{ϵ}-(4-amino-2-hydroxybutyl)lysine). The fact that nature has committed two enzymes to produce one hypusine residue on a single protein underscores the importance of this posttranslational modification. Hypusine formation is tightly coupled to cell proliferation and is essential for cell survival (1–3). Disruption of either the eIF-5A or deoxyhypusine synthase gene in yeast leads to a lethal phenotype (4–6). Inhibition of deoxyhypusine synthase in mammalian cells causes growth arrest (7–9), cell death (10), or tumor differentiation (9). In addition, hypusine formation activity exhibits a marked increase in virally transformed cells (11) but a striking attenuation in senescent cells (12).

Despite the importance of eIF-5A in cell proliferation and survival, the physiological function of this protein is unclear. The notion that eIF-5A is an initiation factor comes from the earlier observations that it can be isolated from the ribosome-bound fraction and that it can stimulate the synthesis of methionyl-puromycin (13–15). However, the role of eIF-5A in translation initiation has been questioned because of a lack of correlation between eIF-5A and general protein synthesis (16–18). Recent studies have suggested that eIF-5A may serve as a target protein for the human immunodeficiency virus type I Rev protein (19) and the human T-cell leukemia virus type 1 Rex protein (20). However, conflicting data have appeared, and direct evidence of interaction between eIF-5A and viral proteins is lacking (21).

X-ray diffraction studies of the eIF-5A precursor from two archaea species show that it is composed of two domains connected by a flexible hinge (22, 23). The N-terminal domain contains the hypusine residue, which carries two positive charges and closely resembles spermidine and spermine. The C-terminal domain consists of five β-strands, which closely resemble the cold shock domain (CSD) present in bacterial cold shock protein A (CspA). These studies suggest that eIF-5A may interact with nucleic acids, particularly RNA. This notion is substantiated by our previous finding that eIF-5A is capable of binding to Rev response element (RRE) and U6 RNA *in vitro* (25) and the finding that the *TIF51A* gene, which encodes eIF-5A in yeast, could complement the temperature-sensitive

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¶ To whom correspondence should be addressed: Dept. of Chemistry, Rutgers-The state University of New Jersey, P. O. Box 939, Piscataway, NJ 08855-0939. Tel.: 732-445-3739; Fax: 732-445-5312; E-mail: KYCHEN@rutchem.rutgers.edu.

[1] The abbreviations used are: eIF-5A, eukaryotic initiation factor 5A; SELEX, systematic evoluton of ligands by exponential enrichment; 6xHis-18K-lys, histidine-tagged human eIF-5A precursor; 6xHis-18K-hyp, histidine-tagged human eIF-5A; RRE, Rev response element; CSD, cold shock domain; CspA, cold shock protein A; hyp, hypusine; PCR, polymerase chain reaction; nt, nucleotide; ss, single-stranded; BSA, bovine serum albumin; EST, expressed sequence tag; UTR, untranslated region.

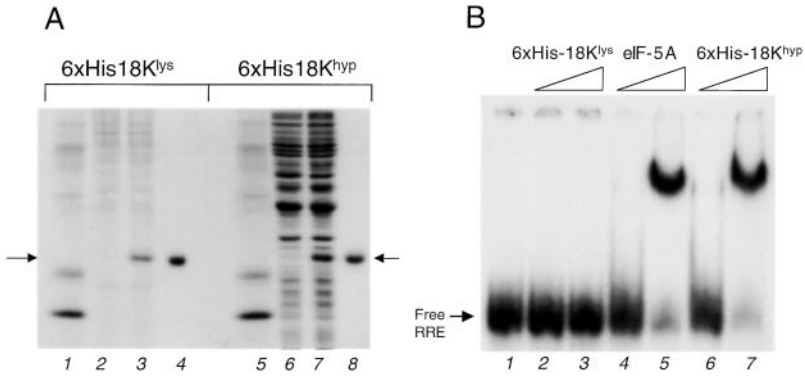

FIG. 1. *A*, purification of unmodified and modified eIF-5A. *Lanes 1* and *5*, protein markers, *lane 2*, flowthrough; *lane 3*, eluent from a Ni-nitrilo triacetic acid column; *lane 4*, purified 6xHis-18K-lys from fast protein liquid chromatography; *lane 6*, uninduced yeast extracts; *lane 7*, induced yeast extracts; *lane 8*, 6xHis-18K-hyp purified from a Ni-nitrilo triacetic acid column and fast protein liquid chromatography (Mono-S). *B*, binding of RRE RNA with 6xHis-18K-lys, HeLa eIF-5A, and 6xHis-18K-hyp. *Lane 1*, RRE RNA alone; *lanes 2* and *3*, RRE RNA plus 6xHis-18K-lys (0.5 and 2.0 μg); *lanes 4* and *5*, RNA probe with HeLa eIF-5A (0.5 and 2.0 μg); *lanes 6* and *7*, RNA probe with modified eIF-5A, 6xHis-18K-hyp (0.5 and 2.0 μg).

growth and mRNA decay phenotypes of ts1159 mutant yeast (24).

In the present study we have used the strategy of systematic evolution of ligands by exponential enrichment (SELEX; Ref. 26) to enrich RNA sequences that bind to eIF-5A with high affinity. We demonstrated that the RNAs enriched over 16 rounds of SELEX shared a high sequence homology, and, more importantly, the binding of eIF-5A to the selected RNAs requires the presence of the hypusine residue.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]UTP and [γ-^{32}P]ATP (3,000 Ci/mmol) were obtained from ICN Chemical Radioisotope Division (Irvine, CA). The yeast expression vector pYES2 was a gift of Drs. Herbert and Celia Tabor (National Institutes of Health). Glutathione *S*-transferase-Rev and pGEM-RRE were given by Dr. Michael H. Malim (University of Pennsylvania), and pET11-CspA and pJJGO2 containing the *CspA* gene were given by Dr. Masayori Inoyue (University of Medicine and Dentistry of New Jersey). All NTPs and RNase inhibitor were purchased from Roche Molecular Biochemicals. Restriction enzymes and other molecular biological supplies including SP6 RNA polymerase and T7 RNA polymerase were from Promega or Amersham Pharmacia Biotech.

Expression of Unmodified and Modified Histidine-tagged eIF-5A—The plasmid pQEh18K containg histidine-tagged the human eIF-5A cDNA insert was constructed from pQTy21 as described previously (27). The histidine-tagged eIF-5A precursor is termed 6xHis-18K-lys, where 18K and lys refer to the apparent molecular weight and the unmodified lysine residue at position 50, respectively. The plasmid pYER18K was constructed by inserting the 6xHis-human eIF-5A cDNA between the *Hin*dIII and *Eco*RI sites of yeast expression vector pYES2. The histidine-tagged eIF-5A is termed 6xHis-18K-hyp, where the lysine 50 residue has been converted to the hypusine residue (hyp). The 6xHis-18K-lys protein was overexpressed in *Escherichia coli* strain BL 21 (DE3), and the 6xHis-18K-hyp protein was overexpressed in yeast (strain 2602). Both 6xHis-18K-lys and 6xHis-18K-hyp were purified by metal affinity chromatography and fast protein liquid chromatography.

SELEX—The oligonucleotide template used for SELEX was first constructed as a single-stranded 98 mer with the following sequence: 5′-GCGGAATTCTAATACGACTCACTATAGGGAACAGTCCGAGCC-$(N)_{40}$GGGTCAATGCGTCATA-3′, where the central 40 base pairs contained a random sequence (N) based on equal incorporation of A, G, C, and T at each position. The complementary strand was synthesized by annealing a primer with the sequence 5′-GCGGGATCCTATGACG-CATTGACCC-3′ (primer 2) followed by the DNA polymerase reaction using a Klenow fragment. Primer 1, containing a T7 RNA polymerase promoter sequence, was used for polymerase chain reaction (PCR) amplification: 5′-GCGGAATCCTAATACGACTCACTATAGGGAACA-GTCCGAGCC-3′. Restriction sites for *Bam*HI and *Eco*RI were included in both primers for cloning. A degenerate double-stranded DNA template was synthesized by PCR using primer 1 (T7 primer) and primer 2 (reverse primer). The template obtained from the PCR reaction was used for *in vitro* transcription to generate a random pool of RNA molecules as described (28). The transcribed RNA was purified by electrophoresis on an 8% polyacrylamide gel. RNA was dissolved in an annealing buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, and 2 mM $MgCl_2$) heated at 95 °C for 2 min and then put on ice for 20 min before the start of the binding reaction. To initiate the *in vitro* selection, the random pool RNAs were first eluted through a 200-μl Ni-nitrilo triacetic acid-resin column (Promega). The eluted RNAs were incubated at 4 °C with 6xHis-18K-hyp (2 μM) in a 20 mM Tris-HCl buffer, pH 8.0, containing 10% glycerol, 1 mM dithiothreitol, 30 mM KCl, 4 mM $MgCl_2$, and 3 μg tRNA for 30 min. The binding mixture was then passed through the Ni-nitrilo triacetic acid-resin column (bed volume 20 μl) and washed with 6 ml of binding buffer containing 0.02 M imidazole and another 4 ml of binding buffer containing 0.05 M imidazole. The bound RNAs were then eluted with the binding buffer containing 0.4 M imidazole. The eluted RNAs were reverse-transcribed by avian myeloblastosis virus reverse transcriptase (20 units; Roche Molecular Biochemicals) using primer 2 in the reverse transcription buffer (Roche Molecular Biochemicals). The cDNAs obtained were then amplified by PCR using primers 1 and 2. The PCR product was purified by electrophoresis through 8% polyacrylamide gels, eluted, and transcribed *in vitro* using T7 RNA polymerase to generate the pool 1 RNAs for the second round of selection. After the 16th selection cycle, the cDNAs obtained from the selected RNAs were cloned into pCR II vector (Invitrogen) for sequence determination.

RNA Preparation and Labeling—The 252-nucleotide (nt) RRE RNA was synthesized by T7 RNA polymerase using linearized pGEM-RRE as the template in the presence of [α-^{32}P]UTP and purified by electrophoresis as described (29). The RNAs with sizes of 23, 44, and 86 nt were synthesized by transcribing of the fragment obtained from restriction enzyme digestion of pBluescript IISK with, respectively, *Xho*I, *Hin*dIII, and *Xba*I. The sequences of these RNAs are: 23 nt, GGC-GAAUUGGGUACCGGGCCCCC; 44 nt, GGCGAAUUGGGUACCGGG-CCCCCCCUCGAGGUCGACGGUAUCG; and 86 nt, GGCGAAUUGG-GUACCGGGCCCCCCCUCGAGGUCGACGGUAUCGAUAAGCUUG-AUAUCGAAUUCCUGCAGCCCGGGGGAUCCACUA. The random RNAs (pre-SELEX RNA) were synthesized using the 98 mer containing N40 as the template. The post-SELEX RNAs were obtained by T7 polymerase transcription of the selected oligonucleotides as template. All the RNA probes were radiolabeled with [α-^{32}P]UTP by T7 RNA ploymerase, and the labeled RNA probes were gel-purified by electrophoresis using a 12% polyacrylamide gel. The labeled RNA was eluted with a solution containing 0.3 M sodium acetate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.5% SDS at 37 °C. For preparation of single-stranded DNA (ssDNA) probes, the specific primer was first labeled with [γ-^{32}P] ATP by T4 kinase and PCR amplification, the labeled PCR products were heated to 100 °C, and ssDNA was separated by electrophoresis on an 8% polyacrylamide gel.

Filter Binding Assay—The filter binding assay was carried out in a final volume of 15 μl binding buffer (10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$ 10 mM KCl, 10% glycerol, 2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin (BSA), 0.5 mg/ml yeast tRNA) containing 6xHis-18K-hyp and fixed amounts of RNA (~50 pM). The mixture was incubated at 4 °C for 20 min. The binding mixture was then filtered through a 0.45-μm BA85 nitrocellulose filter paper (Schleicher & Schuell). The filter paper was thoroughly washed with a solution containing 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, and 10 mM KCl. Radioactivity retained on the filter paper was measured by an LS650 liquid scintillation

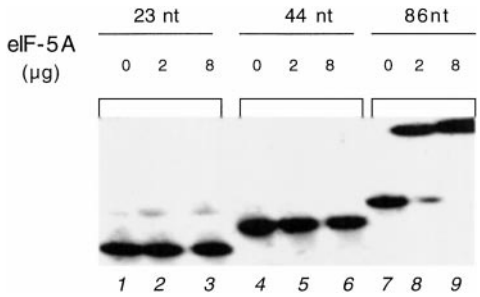

FIG. 2. **Size dependence of the binding of eIF-5A with RNA.** RNA fragments of different lengths were generated by transcribing the pBlueScriptII SK fragments *in vitro* using T7 RNA polymerase. The *Xho*I fragment gives 23 nt; the *Hin*dIII fragment gives 44 nt; and the *Xba*I fragment gives 86 nt. *Lane 1*, RNA (23 nt); *lane 2*, RNA (23 nt) with eIF-5A (2 μg); *lane 3*, RNA (23 nt) with eIF-5A (8 μg); *lane 4*, RNA (44 nt); *lane 5*, RNA (44 nt) with eIF-5A (2 μg); *lane 6*, RNA (44 nt) with eIF-5A (8 μg); *lane 7*, RNA (86 nt); *lane 8*, RNA (86 nt) with eIF-5A (2 μg); *lane 9*, RNA (86 nt) with eIF-5A (8 μg).

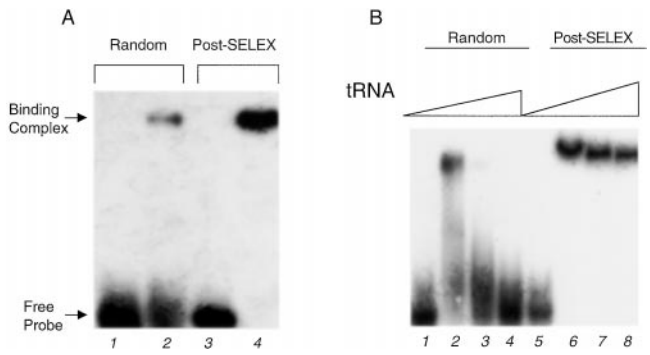

FIG. 3. **Binding of 6xHis-18K-hyp with random and post-SELEX RNA.** The ^{32}P-labeled RNA probe (10,000 cpm) was incubated with eIF-5A, and the binding complex was resolved on a native polyacrylamide gel (8%; acrylamide/bis-acrylamide = 29) by electrophoresis. Positions of free RNA and protein-RNA complex are shown by *arrows*. *A, lane 1*, random RNA alone; *lane 2*, random RNA with 6xHis-18K-hyp (2 μg); *lane 3*, post-SELEX RNA; *lane 4*, post-SELEX RNA with 6xHis-18K-hyp (2 μg). *B*, *lane 1*, random RNA alone; *lanes 2–4*, random RNA with 6xHis-18K-hyp (2 μg) in the presence of *E. coli* tRNA (0.2, 2, and 20 μM, respectively); *lane 5*, post-SELEX RNA alone; *lanes 6–8*, post-SELEX RNA with 6xHis-18K-hyp (2 μg) in the presence of *E. coli* tRNA (0.2, 2, and 20 μM, respectively).

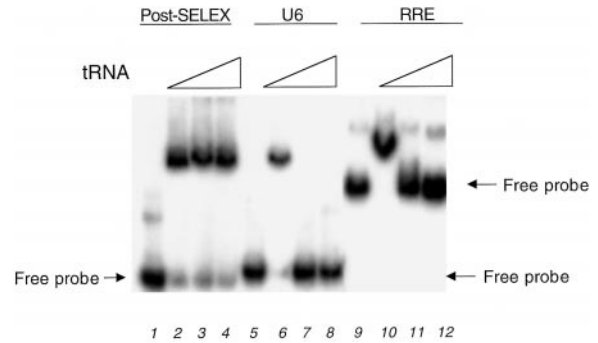

FIG. 4. **Binding of 6xHis-18K-hyp with post-SELEX RNA, U6 RNA, and RRE RNA.** The ^{32}P-labeled RNA probe (10,000 cpm) was incubated with 6xHis-18K-hyp at a final concentration of 4 μM, and the binding complex was analyzed by electrophoresis on a native polyacrylamide gel (8%; acrylamide/bis-acrylamide = 29). *Lane 1*, Post-SELEX RNA alone; *lanes 2–4*, Post-SELEX RNA probe with 6xHis-18K-hyp in the presence of increasing amounts of *E. coli* tRNA (0.2, 2, and 20 μM, respectively); *lane 5*, U6 RNA alone; *lanes 6–8*, U6 RNA with 6xHis-18K-hyp in the presence of increasing amounts of *E. coli* tRNA (0.2, 2, and 20 μM, respectively); *lane 9*, RRE RNA alone; *lanes 10–12*, RRE RNA with 6xHis-18K-hyp in the presence of increasing amounts of *E. coli* tRNA (0.2, 2, and 20 μM, respectively).

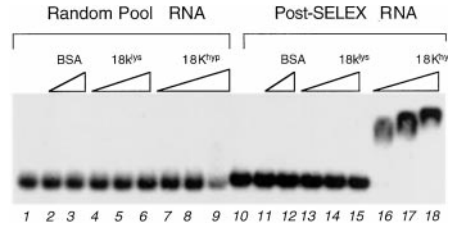

FIG. 5. **The hypusine residue is required for eIF-5A-RNA binding.** Radiolabeled and gel-purified random RNA or post-SELEX RNA was incubated with BSA, 6xHis-18K-lys, or 6xHis-18K-hyp at different concentrations. The reaction mixtures were then analyzed by gel mobility shift assay on an 8% native gel. *Lane 1*, random RNA probe alone; *lanes 2* and *3*, random RNA with BSA (5 and 10 μM); *lanes 4–6*, random RNA with 6xHis-18K-lys (7, 15, and 30 μM, respectively); *lanes 7–9*, random RNA with 6xHis-18K-hyp (7, 15, and 30 μM, respectively); *lane 10*, post-SELEX RNA probe alone; *lanes 11* and *12*, post-SELEX RNA with BSA (5 and 10 μM); *lanes 13–15*, post-SELEX RNA probe wih 6xHis-18K-lys (7, 15, and 30 μM, respectively); *lanes 16–18*, post-SELEX RNA probe with 6xHis-18K-hyp (7, 15, and 30 μM, respectively). *18K^{lys}*, 6xHis-18K-lys; *18K^{hyp}*, 6xHis-18K-hyp.

counter. The retention of free RNA (always <10%) was substracted from all data points.

Gel Mobility Shift Assay—Radioactively labeled RNA or ssDNA (~10 fmol) was incubated with 6xHis-18K-hyp or 6xHis-18K-lys protein for 15 min on ice in 15 μl of binding buffer (10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 10 mM KCl, 10% glycerol, 2 mM dithiothreitol, 0.25 mg/ml BSA, yeast tRNA at indicated concentrations). The stringent conditions referred to the binding reactions carried out in the presence of high tRNA concentrations (>2 μM). The binding mixture was loaded onto an 8% polyacrylamide gel made in Tris borate-EDTA buffer containing 5% glycerol, and electrophoresis was carried out at 4 °C. After the electrophoresis the gel was vacuum-dried and visualized by autoradiography.

Ribonuclease Protection Assay—RNA probes were radiolabeled with [α -P^{32}]UTP during *in vitro* transcription. The labeled RNA was incubated with various amounts of purified 6xHis-18K-hyp or other recombinant proteins in the RNA binding buffer for 15 min on ice. RNase T1 or RNase A was then added to the binding mixture to initiate RNA digestion. The reaction mixture was kept on ice for 15 min and analyzed by electrophoresis on a 12% polyacrylamide gel. The gel was fixed and dried on a DE-81 paper for autoradiography.

RESULTS

Binding of Histidine-tagged eIF-5A to RRE RNA—Fig. 1*A* shows the purification of the recombinant eIF-5A precursor (6xHis-18K-lys) from *E. coli* and the recombinant eIF-5A (6xHis-18K-hyp) from yeast (Fig. 1*A, lanes 4* and *8*). The RNA binding activity of these two recombinant proteins was compared with that of the wild type HeLa eIF-5A. Fig. 1*B* shows that HeLa eIF-5A and recombinant eIF-5A 6xHis-18K-hyp, but not the eIF-5A precursor 6xHis-18K-lys, could bind to RRE RNA (Fig. 1*B, lanes 5* and *7 versus lane 3*), indicating that the histidine tag did not interfere with the RNA binding activity of recombinant eIF-5A. We therefore could use 6xHis-18K-hyp affinity chromatography to initiate the *in vitro* selection.

Size Requirement of RNA for eIF-5A Binding—To determine the optimal size of RNA required for *in vitro* selection, we have tested the binding of eIF-5A to RNAs of different sizes under nonstringent conditions. Fig. 2 shows that 6xHis-18K-hyp did not bind to the 23- or 44-nt RNA (Fig. 2, *lanes 1–6*) but could bind to the longer RNA with a length of 86 nt (Fig. 2, *lanes 8* and *9 versus lane 7*). On the basis of this result, we designed a DNA template with a total length of 98 nt, consisting of a variable region of 40 nt (N40) and a 5′-end-flanking region containing the T7 RNA polymerase promoter. Both 5′- and 3′-end-flanking regions were designed to avoid self-complementarity and to minimize secondary structure formation that may bias the selection (30).

In Vitro Selection of RNA Specifically Recognized by Human eIF-5A—The 98-nt template was used to generate a DNA pool consisting of ~10^{24} unique sequences. The DNA pool was transcribed *in vitro* to give a random RNA pool, termed pre-SELEX

TABLE I
Sequence determination of post-SELEX and pre-SELEX RNA

Clone	Sequence
Sequences of post-SELEX RNA clones	
1	AGCTATGACTCCTAAAACCACGCGCCTCGCAATGTCACAC
2	AGCTAGTACTCCCTAACCATGTGCCGTCTAAATCTCACAC
3	AGCTAGTAACTCCCTAACCCGCGCCTGCTAAATGTCACAC
4	AGGCAAATACTTCCTAACCACGCCGCTCAAAATGTCACAC
5	AGCGAATACTTCCTAACCACGCGCCTGCTAAATGTCACAC
6	AGCTAGTACTCCCTAACCACGCGCCTGCTAAATGTCACAC
7	AGCTAGTACTTAACCACGCGCCTCGTAAATGTCACAC
8	GCTAGTACTCCTAACCACCTTAAATGTCACACCCGAG
9	AGCATAGTACTTAACCACGCGCCTCGAAATGTCACAC
10	AGCGAATACTTCCTAACCACGCGCCTGCTAAAGTCACAC
11	AGCAGTACTCCCTAACCAACGCGCCTGCTAAATGTCACAC
Sequences of pre-SELEX RNA clones	
a	ATATAGCTTAAGTGCTAAATACATTCCGTGACTTGCAGA
b	ACTCAGACATACGCTAAGGTTTAATTAGCCTACAAGGTCAAATT
c	TGGATAACTAAATTTGATGAAAAGTTAGTTTGGGTT
d	GAGCCGCTAGTAATTGCCTGTACCTGTGCTTACGTGCGAAGA
e	CCTTCTGTCCAATATCCTGAGTCTTAGCTTA
f	GTCCATTAAGATACCAGTTAGGTAATCTAGGGTTGTTAGC
g	GTGATCGAGATAGATTCGAGTAAAGAATTCGATAAAATGGGACGGGCC

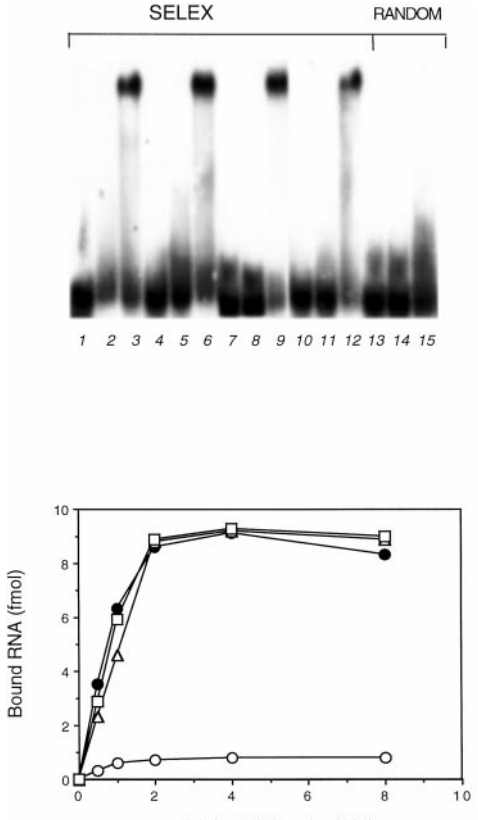

FIG. 6. **Binding characteristics of 6xHis-18K-hyp with cloned post-SELEX RNA.** *A*, gel mobility shift assay of the binding between 6xHis-18K-hyp and independently cloned post-SELEX RNA. *Lane 1*, clone 1 RNA alone; *lanes 2* and *3*, clone 1 RNA with 6xHis-18K-hyp (0.7 and 3.5 μM); *lane 4*, clone 2 RNA alone; *lanes 5* and *6*, clone 2 RNA with 6xHis-18K-hyp (0.7 and 3.5 μM); *lane 7*, clone 3 RNA alone; *lanes 8* and *9*, clone 3 RNA with 6xHis-18K-hyp (0.7 and 3.5 μM); *lane 10*, clone 4 RNA alone; *lanes 11* and *12*, clone 4 RNA with 6xHis-18K-hyp (0.7 and 0.5 μM); *lane 13*, random RNA probe; *lanes 14* and *15* random RNA with 6xHis-18K-hyp (0.7 and 3.5 μM). *B*, filter paper binding assay. The filter paper binding assay was carried out using variable amounts of 6xHis-18K-hyp with a fixed amount of ^{32}P-labeled random RNA probe (×) or post-SELEX RNA clones (○, □, and △).

RNA. The selection was initiated with the random RNA pool, using 6xHis-18K-hyp as the bait protein (round 0). The RNA pool enriched after 16 cycles of selection was termed post-SELEX RNA. Post-SELEX RNA was then tested for binding with 6xHis-18K-hyp using a gel mobility shift assay. Fig. 3*A* shows that the binding affinity of 6xHis-18K-hyp for the post-SELEX RNA was ~10-fold greater than that for random RNA under nonstringent conditions (Fig. 3*A, lane 4 versus lane 2*). However, the difference in binding affinity became quite pronounced under a more stringent binding condition. Thus, with a higher concentration of tRNA (>2 μM) in the binding buffer, the binding of eIF-5A to random RNA was completely abolished (Fig. 3*B, lanes 3* and *4 versus lane 2*). In contrast, the binding between eIF-5A and post-SELEX RNA remained intact even in the presence of 20 μM tRNA (Fig. 3*B, lane 8*). We also compared the binding of eIF-5A to RRE and U6 RNA with that to post-SELEX RNA. Fig. 4 shows that eIF-5A bound to all three RNAs equally well under nonstringent conditions (Fig. 4, *lanes 2, 6*, and *10*). However, under more stringent conditions, eIF-5A bound only to post-SELEX RNA, but not RRE or U6 RNA (Fig. 4, *lanes 8* and *12 versus lane 4*). Although the binding of eIF-5A to RRE or U6 RNA appeared to be less specific, we cannot rule out the possibility that such binding could still serve some useful functions *in vivo*.

Role of Hypusine Residue in the Binding of eIF-5A to Post-SELEX RNA—To determine the role of hypusine in RNA binding, we compared the RNA binding activity of eIF-5A (6xHis-18K-hyp) with that of its precursor (6xHis-18K-lys). Fig. 5 shows that both BSA, an unrelated protein, and 6xHis-18K-lys did not exhibit any binding activity with either random or the post-SELEX RNA (Fig. 5, *lanes 2–6* and *11–15*). In contrast, 6xHis-18K-hyp exhibited strong binding with the post-SELEX RNA but not with random RNA (Fig. 5, *lanes 16–18 versus lanes 7–9*). Because the difference between 6xHis-18K-hyp and 6xHis-18K-lys is limited to only one amino acid residue, namely, hypusine *versus* lysine, the striking difference in their binding activities toward post-SELEX RNA argues strongly for the important role of hypusine in the eIF-5A-RNA interaction.

Sequences and Binding Affinity of Cloned Post-SELEX RNA—To characterize the nature of post-SELEX RNA, we have isolated independent clones from both pre- and post-SELEX RNA pools for sequence determination and binding study. Table I lists the sequences of these RNA clones. The individual post-SELEX RNA clones exhibited strong binding to eIF-5A under stringent conditions (Fig. 6*A, lanes 3, 6, 9*, and *12*). Assuming a 1:1 binding stoichiometry, the apparent K_d value for the eIF-5A-RNA binding complex was estimated to be $\sim 1 \times 10^{-7}$ (Fig. 6*B*). The apparent K_d value for the binding of

eIF-5A to random RNA was difficult to estimate because of the low binding affinity under the stringent conditions. The maximal level of binding of eIF-5A with post-SELEX RNA was >100-fold higher than that with pre-SELEX RNA.

Consensus Sequences and BLAST Analysis—With the sequences of post-SELEX RNA available (Table I), we proceeded to examine whether they shared any sequence homology. Fig. 7 shows that, on the basis of sequence alignment, the consensus sequence of post-SELEX RNA can be defined as either CCUAACCACGCGCCU (sequence I) or CUAAAUGUCACAC (sequence II). If gap formation is allowed, the consensus sequence can also be defined as sequence I + II (CCUAACCACGCGCCUnnCUAAAUGUCACAC). To determine which one is important for eIF-5A binding, we have generated four additional 98-nt RNA sequences (Table II, probes 2–5) containing either sequence I or sequence II and compared their binding affinity to eIF-5A with that of post-SELEX RNA. Table II shows that RNAs containing sequence II (probes 4 and 5) retained high binding affinity with eIF-5A, whereas RNAs containing sequence I (probes 2 and 3) exhibited reduced binding affinity with eIF-5A, indicating that sequence II is required for high affinity eIF-5A binding. Because the two RNAs containing sequence II at different positions exhibited similar binding affinity to eIF-5A (Table II, probe 4 *versus* probe 5), the position of sequence II within the 98-nt RNA sequence may not be crucial for eIF-5A binding. We also generated a 40-nt RNA with the sequence identical to that of the variable region (N40) of the clone 6 post-SELEX RNA. We found that the binding affinity of eIF-5A with the 40-nt RNA was only ~1/10 of that obtained with the 98-nt post-SELEX RNA (data not shown), suggesting that the flanking regions are likely to contribute to the overall stabilization of the eIF-5A-RNA complex.

Next we searched for a possible occurrence of sequences I and II in the GenBank or EST data base by BLAST analysis. We found that the 11-nt sequence I (AACCACGCGCCU) is present in 53 EST sequences, and the 11-nt sequence II (AAAUGUCACAC) is present in 474 sequences. However, no match could be found for the 30-nt sequence I + II in either the GenBank or EST data base. Although the sequence II motif appears to be important in the sequence-specific binding of eIF-5A, it remains to be investigated whether the EST sequences that contain sequence II could serve as the physiological targets of eIF-5A *in vivo*. Along this line, we are currently using the specific nucleic acids associated with proteins method (31) to identify the physiological ligands of eIF-5A.

Binding of 6xHis-18K-hyp to ssDNA—Most CSD-containing proteins are capable of binding to ssDNA (32). We therefore examined whether eIF-5A could also interact with ssDNA. Both sense and antisense single-stranded DNA were generated from random and post-SELEX RNA clones for gel mobility shift assay. Fig. 8 shows that eIF-5A could bind to the sense ssDNA derived from post-SELEX RNA (Fig. 8, *lane 7*), similar to CspA (Fig. 8, *lanes 2* and *3 versus lane 1*). However, when eIF-5A was used at a lower concentration, the mobility of ssDNA was only partially retarded and appeared as a diffused band (Fig. 8, *lane 6, band a*). We suspected that the band was attributable to the interaction of CSD with ssDNA, because: (i) a similar diffused band was apparent when the eIF-5A precursor was used in the binding assay (Fig. 8, *lanes 4* and *5, band a*); and (ii) the C-terminal half of eIF-5A (eIF-5A 84–154) could partially retard the ssDNA (Fig. 8, *lane 8*). The antisense ssDNA from either random or post-SELEX RNA or the sense ssDNA derived from pre-SELEX RNA did not bind to eIF-5A under the same binding conditions (data not shown). These data suggest that the eIF-5A-ssDNA binding was sequence-specific.

Functional Assay—Because eIF-5A shares a certain structural similarity with CspA (22, 23), it is tempting to speculate that they may share some functional similarity. CspA has been proposed to function as an RNA chaperon because it facilitates the ribonuclease-catalyzed degradation of 5′-untranslated region (UTR) CspA RNA (32). It is therefore of interest to test whether eIF-5A shows a similar effect in the same assay system. A direct comparison of the effect of eIF-5A with that of CspA on the 5′-UTR degradation seems warranted, because both proteins appeared to bind to the 5′-UTR equally well under the experimental conditions (Fig. 9, *lanes 2* and *5 versus lane 1*). Whereas the binding of CspA to the 5′-UTR could facilitate the degradation of the RNA (Fig. 9, *lane 4 versus lane 3*), as previously reported (32), the binding of eIF-5A to the 5′-UTR did not alter the sensitivity of the RNA to RNase T1 (Fig. 9, *lanes 9–11 versus lanes 6–8*). We then performed a similar ribonuclease assay using the post-SELEX RNA as the

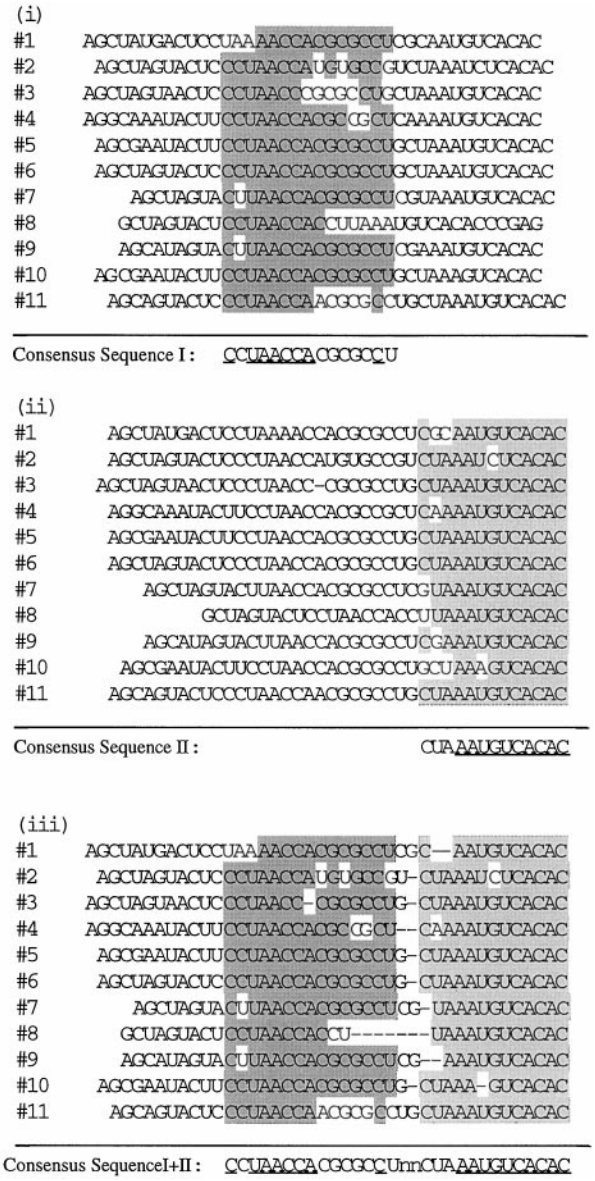

FIG. 7. **Sequence alignment of post-SELEX RNA.** The conserved region was identified by sequence alignment and is indicated by *shading*. In case *(iii)* sequence alignment was performed by the Genetics Computer Group Pileup program with gap formation allowed for maximal alignment. The consensus sequence in each case was determined on the basis of the frequency of appearance of a specific nucleotide. The nucleotides that appeared with a frequency ≥64% are indicated. Nucleotides that appear with a frequency >90% are *underlined*. *n,* no specific nucleotide appears at that position with a frequency >50%.

TABLE II
Binding affinity of RNAs with consensus sequences I, II, and I + II with eIF-5A

Probe	The N40 sequences of the RNA probes[a]	Relative binding affinity
1	AGCUAGUACUCCC<u>UAACCACGCGC</u>CUGCU<u>AAAUGUCACAC</u> (I, II)	100
2	AGCUAGUACUCCC<u>UAACCACGCGC</u>CUGCU*GGGCUCUGUGU* (I)	30–40
3	AGCUAGUACUCCC*CGGUUGUAUAU*CUGCU<u>UAACCACGCGC</u> (I)	30–40
4	AGCUAGUACUCCC*CGGUUGUAUAU*CUGCU<u>AAAUGUCACAC</u> (II)	95–100
5	AGCUAGUACUCCC<u>AAAUGUCACAC</u>CUGCU*GGGCUCUGUGU* (II)	95–100
6	AUAUAGCUUAAGUGCUAAAUACAUUCCGUGACUUGCAGA	0–5

[a] All the RNA probes were *in vitro*-transcribed and labeled as described under "Experimental Procedures." All the RNA probes have the identical 5′- and 3′-flanking sequences. The sequences of the N40 region are listed. Sequences I and II in each RNA probe are underlined and indicated. In probes 3 and 4, the position for sequence I was replaced by a random sequence, *CGGUUGUAUAU* (italics). In probes 2 and 5, the position for sequence II was replaced by a random sequence, *GGGCUCUGUGU* (italics). Filter paper binding assay was performed as described under "Experimental Procedures." Probe 1 is identical to post-SELEX clone 6, and probe 6 is a pre-SELEX clone.
[b] The relative binding affinity is defined as the maximal binding relative to that of the post-SELEX RNA (clone 6).

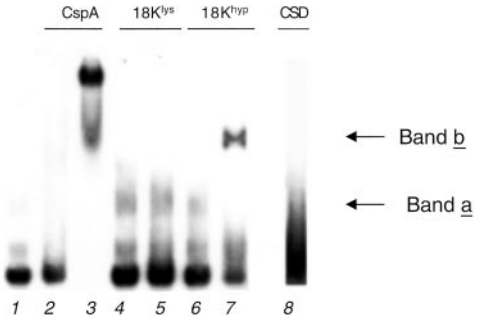

FIG. 8. **The binding of eIF-5A with ssDNA.** ssDNA was prepared as described under "Experimental Procedures." *Lane 1*, ssDNA only; *lanes 2* and *3*, ssDNA with CspA (0.5 and 3.0 μg); *lanes 4* and *5*, ssDNA with 6xHis-18K-lys (0.5 and 3 μg); *lanes 6* and *7*, ssDNA with 6xHis-18K-hyp (0.5 and 3 μg); *lane 8*, ssDNA with CSD (eIF-5A 84–154; 3 μg). The binding mixture was analyzed by electrophoresis on an 8% polyacrylamide gel. *Bands a* and *b,* retarded protein-DNA complexes.

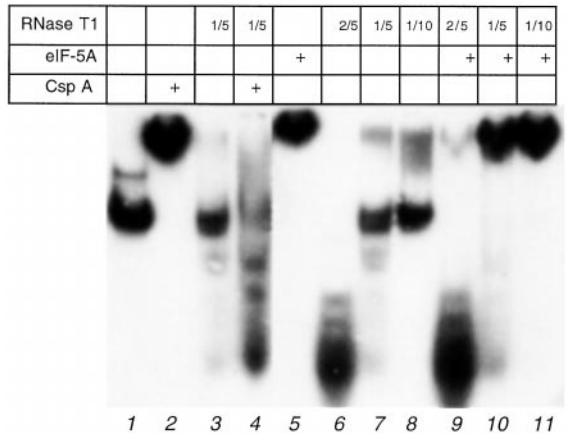

FIG. 9. **Effects of CspA and eIF-5A on the RNase T1-catalyzed degradation of 5′-UTR CspA mRNA.** Ribonuclease assay was performed as described under "Experimental Procedures." The RNA substrate was derived from the 5′-UTR of the CspA mRNA with the region from +1 to +142 placed under a T7 promoter. The RNA was labeled with with [α-^{32}P]UTP. The RNase assay was carried out at 15 °C for 10 min. The amount of RNase T1 is shown in units. The protein CspA or 6xHis-18K-hyp at 3 μg/assay was added (+) with the RNA before RNase digestion. The final reaction volume was 15 μl. *Lane 1*, RNA substrate alone; *lane 2*, RNA with CspA; *lane 3*, RNase T1 (1 unit) was added to RNA; *lane 4*, RNase T1 (1 unit) was added to RNA with CspA; *lane 5*, RNA with 6xHis-18K-hyp; *lanes 6–8*, 10, 1, and 0.1 units of RNase T1, respectively, added to RNA; *lanes 9–11*, 10, 1, and 0.1 units of RNase T1, respectively, added to RNA with 6xHis-18K-hyp. Immediately after the reaction, the reaction mixture was loaded onto an 8% acrylamide gel, and electrophoresis was carried out at 150 V at 4 °C.

target RNA. Fig. 10 shows that the ribonuclease digestion patterns of post-SELEX RNA with either RNase A or RNase T1 were almost identical whether measured in the absence or in the presence of eIF-5A (Fig. 10, *lanes 7–10 versus lanes 3–6* and *lanes 15–18 versus lanes 11–14*). These results suggest that eIF-5A may not functionally resemble CspA, at least under the present assay system.

DISCUSSION

eIF-5A is the only cellular protein known to contain hypusine (1–3). Although eIF-5A has been shown to be essential for growth and proliferation, its precise physiological function is still unclear. On the basis of the finding that eIF-5A can bind to RRE and U6 RNA, we have proposed that eIF-5A may function as an RNA-binding protein (25). In the present study we have used SELEX, an *in vitro* selection method, to enrich and identify RNAs that may specifically bind to eIF-5A. The enriched RNAs, termed post-SELEX RNAs, shared a high sequence homology (Table I and Fig. 7) and showed enhanced binding affinity with eIF-5A (Fig. 6). The binding specificity between eIF-5A and post-SELEX RNA was quite high, as indicated by the observation that the binding occurred even in the presence of 20 μM tRNA (Figs. 3 and 4). The finding that eIF-5A can bind to RNA in a sequence-specific manner suggests that eIF-5A may indeed function as an RNA-binding protein. More importantly, the binding of eIF-5A to the selected RNA requires the presence of a hypusine residue (Fig. 5), suggesting that hypusine has a role in the eIF-5A-RNA interaction and that the hypusine-dependent RNA binding might be biologically relevant. The notion that eIF-5A may selectively affect the expression and/or degradation of a small group of certain mRNAs (18, 24) is consistent with possibility that some of the EST sequences that contain sequence II may serve as the physiological ligands of eIF-5A.

X-ray structures of archaebacterial eIF-5A reveal the presence of hypusine at the N-terminal domain and CSD in the C-terminal domain (22, 23). Although both the hypusine site and CSD have the potential to interact with RNA, the finding that eIF-5A, but not the eIF-5A precursor, binds to post-SELEX RNA suggests that the hypusine site may be more critical than CSD in the sequence-specific eIF-5A-RNA interaction. In this regard, it may not be surprising that eIF-5A behaves differently from CspA, the namesake of CSD, in the ribonuclease protection assay (Figs. 8 and 9). However, we cannot rule out the possibility that eIF-5A may still function as an RNA chaperon with certain other target RNAs.

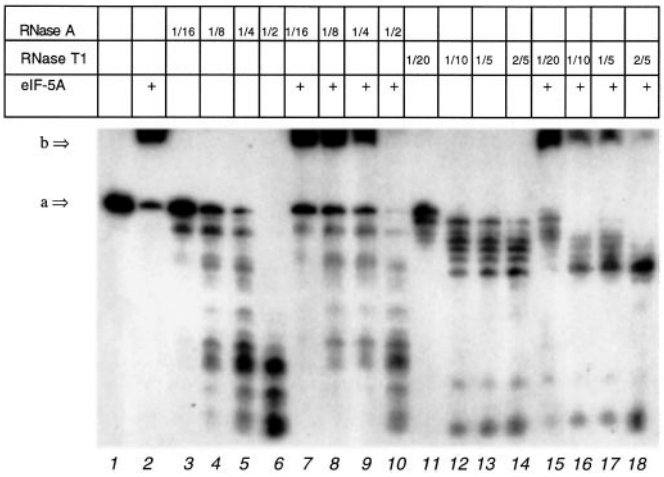

FIG. 10. **Effect of eIF-5A binding on the degradation of RNA catalyzed by RNase T1 or RNase A.** Ribonuclease assay was performed as described under "Experimental Procedures." The cloned post-SELEX RNA (clone 3) was radiolabeled with [α-^{32}P]UTP and used as the substrate. The reaction mixture (final volume of 15 μl) was incubated for 10 min at 15 °C. The amount of ribonuclease is shown in μg. The eIF-5A protein, 6xHis-18K-hyp, was added at the amount of 3 μg/assay before the addition of RNase T1 or RNase A. *Lane 1*, clone 3 RNA alone; *lane 2*, RNA plus 6xHis-18K-hyp; *lanes 3–6*, 0.063, 0.125, 0.25, and 0.5 μg of RNase T1, respectively, added to the reaction mixture; *lanes 7–10*, same as *lanes 3–6*, except that 6xHis-18K-hyp (3 μg) was first mixed with RNA; *lanes 11–14*, 0.05, 0.1, 0.2, and 0.4 μg of RNase A, respectively, added to the reaction mixture; *lanes 15–18*, same as *lanes 11–14*, respectively, except that 6xHis-18K-hyp (3 μg/assay) was first mixed with RNA. Immediately after the reaction, the reaction mixture was applied to a 12% polyacrylamide gel, and electrophoresis was carried out at 150 V at 4 °C.

RNA-binding proteins such as Rev and NS1 are bimodular in that they have a nucleic acid binding domain containing a basic amino acid cluster at the N terminus and a leucine-rich domain at the C terminus used for protein interaction (33). Motif analysis shows the presence of a stretch of basic amino acids clustered at the hypusine site and a leucine-rich stretch at the C-terminal domain in eIF-5A (25). Thus, instead of binding only with RNA, eIF-5A may use its bimodular structure to interact with both RNA and protein. Indeed, eIF-5A has been shown to interact with deoxyhypusine synthase (35), transglutaminase II (36), L5 (37), CRM1 (38), and exportin 4 (39). It remains to be determined whether any of these proteins is truly the physiological substrate of eIF-5A. Nevertheless, these studies and the finding that eIF-5A can bind to RNA in a sequence-specific manner support the notion that eIF-5A is a bimodular RNA-binding protein. RNA-binding proteins have diverse functions related to RNA processing, turnover, nucleocytoplasmic transport, and transcription and translation (33, 34). Although the precise functions of eIF-5A *in vivo* have been difficult to define, the possibility that eIF-5A may function as an RNA-binding protein provides useful clues on the physiological functions of eIF-5A in living cells.

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